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A RE-EVALUATION OF THE INTRA-ARTERIAL METHYLENE BLUE (METHOD OF EHRLICH) IN STUDIES OF THE CENTRAL NERVOUS SYSTEM *

by

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INTRODUCTION

Intra-arterial methylene blue in vivo, known as the method of EHRLICH, was first used by RAMÓN y CAJAL as an analytic stain for studies of the central nervous system (CNS) with success. His remarkable results together with the uncomparable potentiality of the method therein are seen through the beautifully hand-sketched illustrations appearing in the classical textbook (RAMÓN y CAJAL, '11) as well as in its original article (RAMÓN y CAJAL, 1896).

Strangely enough, however, scant literatures are available when informations as to further repeatance or additional progress of the method for CNS were looked for, showing clear-cut contrast with that numberless works in the peripheral nervous system so far done have received much of the impetus from the method (see for references: DOGIEL, '26; WOOLLARD et al., '40; POLYAK, '41).

Whatever the reasons thereupon might have been, it seems timely and worthy of repeating RAMÓN y CAJAL's results first of all and of locating the space, if at all, where the method might be virtually in possession among or in addition to other stains for CNS from a contemporary view point.

Moreover, in view of the fact that the exact implications of RAMÓN y CAJAL's statement as to his having had to discard methylene blue from degeneration study (RAMÓN y CAJAL, '28) need to be examined when judged from the data upon

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peripheral nerves (for examples: WEDDELL and GLEES, '41; FUKUDA, '53), an exploration along the line will be also implied in this study. A supportive statement favoring for an attempt of the kind can be found (FEINDEL, ALLISON and WEDDELL, '48).

PROCEDURE

After certain number of examinations upon those described by various workers (BETHE, 1896; DOGIEL, '26; POLYAK, '41 and RAMÓN y CAJAL, 1896), it was learned empirically that results were best obtained in fair constancy by employing a rather simplified procedure as follow, owing to that of the WEDDELL School's (FEINDEL et al., '48).

As material, young adult albino rats of both sexes weighing about 100-200 g were used. 4 of 3 weeks old and 2 of adult rabbits were also available.

1. Dye: Methylene blue chloride (MERCK) was used exclusively. Several different samples of the dye selected arbitrarily were found without noticeable differences in their results.
2. Injection: 2.5% of the dye in normal saline was injected through the exposed common carotid or aorta of animal anesthetized by using ether with or without combination of nembutal. The amount of the solution to inject ran usually 3-5cc per 100 g of body weight. The speed of the injection* was empirically learned to adjust so as to finish the total dose within 5-10 seconds following respiratory arrest of the animal.
3. Fixation for the dye: Following careful removal, material was cut with a sharp razor blade or the like into smaller tissue blocks with each thickness not more than 5-8 mm. They were kept in freshly prepared 6% ammonium molybdate-saline solution for 3 to 12 hours accordingly to their thicknesses.
4. Tissue fixation: The blocks were then washed in chilled normal saline for 5-10 minutes with three changes and transfered into 10% of neutral formalin for 24 hours up to 2 days.

Blocks thus prepared are ready for frozen sections, paraffin and celloidin sections as well, though the first was used exclusively during this study throughout. Only caution further to take is to pass through the graded alcohols as quickly as possible by having the slides dry in the air or the like, in order for the dye not escape.

RESULTS

As shown in the attached figures (Fig. 1-11 and 13-16), most of potentially important neural elements hitherto known are, in fundamental correspondence with the results of RAMÓN y CAJAL, well demonstrated with the method hereby employed. Under microscope, these neurones can be followed to a considerable distance so that

* Question has been raised if such an injection is not breaking finer capillaries of brain when the pressure at the time of injection becomes reasonably strong accordingly to the vital conditions of animal. Although no direct evidences to rule out the point are in hands, Professor DEMPSEY has indicated an optimistic support as to the question by saying that basic dyes pass through so called blood-brain-barrier (DEMPSEY, '56).

their three dimensional relationships together with fairly delicate connections thereupon become sufficiently clear. Moreover, finer structures such as cellular inclusions, nuclei, branching modes of dendritic processes, pericellular neurofibrils et neuropils and boutons terminaux et de passages can be observed in high constancy. Significance of such demonstrations will be readily understood if those investigations upon both experimental and theoretical central neurophysiology (for instances; BISHOP, '55; ECCLES, '53; LORENTE de NÓ, '43; O'LEARY, '49; WIENER, '48), from which a good number of studies going on currently in the field have obtained the impetus in various way, be remembered.

These stained neural elements can be usually increased in their number to a considerable extent when the temperature of dye-solution was so adjusted closely to that of body, that is 30-35 °C at the time of injection. This also increases the number of glial elements and other connective tissues, the elements otherwise remaining relatively or completely unstained. In addition, these cells thus become stained are usually but not always found green, in contrast with those stained blue.

It is noteworthy that the constancy of these results has been high in various areas of subcortical structure including spinal cord and less so in both cerebral and cerebellar cortex, due presumably to the anoxia, an unavoidable condition present at the time of injection.*

STUDY FOR THE DEGENERATING FIBRES

"Since the aforementioned procedure has been satisfactorily constant in regard to the subcortical structure in general on control animals, the primary optic center was chosen for the alleged purpose. Relevancy of the choice together with the adequacy of the set up described below has been properly explained elsewhere (FEINDEL et al., '48).

1. Material and procedure: Young adult rats with the descriptions otherwise same as those for the control study were used. Removal of unilateral eye was performed with aseptic and antiseptic cautions in the manner essentially similar to that of FEINDEL et al.

At various intervals of 24 hours up to 30 days following the operation, animals were sacrificed by the very procedure used for controls. Preparations were made again for the routine frozen sections of 30 to 80 microns.

2. Result: Unexpectedly, results were disturbed to almost fatal extent by intensive blue staining of the every optic center side opposite to the lesion, so intensively dark enough that degenerating elements expected were hardly identified under microscopic examination. This might have been due partly to vascular abnormality caused by the operation, intact vascularization playing an indispensable rôle for the employed procedure, or else due to some unknown factors yet uncalculated.

* Various ways of combining acetylcholine, glucose, alcohol and hyaluronidase with or without the dye fixation by way of intraarterially injecting ammonium molybdate solution, the techniques having been suggested by various workers (see for references: HELLER et al., '47; MCGREGOR, '53), have so far shown no noticeable advantages to the point.

Among those, however, there were slides, though few, in which certain characteristics assignable to the degenerating changes were observable. They showed the typical disintegration of structure, consisted of irregularly swollen bundle with or without ballooned vesicles or node formations, stained usually more intensively compared with the control side (see Fig. 12, 17, 18 and 19). Although these changes were present as early as in 24 hours following the operation, various stages of these were best found in animals of 3-5 days postoperative. In agreement with the confirmation reported by the WEDDELL School (FEINDEL et al., '48) and that of MEADER by MARCHI method (MEADER, '39), these changes appeared to be more advanced toward the termination of the optic tract, that is in areas most remote from the lesion.

In two cases, metachromatically stained fibres were seen as the exception. They were violet blue (see Fig. 8) or purplish blue and stained so both intact and degenerating fibres as well.

DISCUSSION

1. On the control study: Results shown indicate that a good part of the results obtained by RAMÓN y CAJAL has been satisfactorily repeated by use of the somewhat modified method of EHRLICH developed during this study. Because of their close resemblances in some details, the method may well have the chance as a method of choice for substituting the GOLGI, the rather time consuming and uncertain technique as known. Emphasis with the same view point has been already made by POLYAK through his fruitful work on the retina (POLYAK, '41). It will be hazardous if the foregoing is subjected to a serious discussion, since it is yet empirical matter as ever, remembering that RAMÓN y CAJAL was only one successful among his contemporaries (RAMÓN y CAJAL, '37). That in our laboratory the method has been otherwise applied as a general stain with little modification for histologically localizing acutely and chronically implanted electrodes in CNS (FUKUDA, '57), will be therefore only remaining as an indication in favor of the affirmative answer.

In addition, it may be of some interest to just point out that some of available data indicates further possibility of the method upon the currently developing field in neurochemistry (see: WISLOCKI and DEMPSEY, '48; WORLEY and WORLEY, '43) in the light of a pertinent criticism along the line raised by POPE (POPE, '55).

2. On the central degeneration study: Data are, as shown, inconclusive neither for nor against the statement of RAMÓN y CAJAL. However, the characteristic changes observed seem, basing upon the experience (FUKUDA, '53), to be indicating for further critical studies to be done. That the interesting report done by the WEDDELL School (FEINDEL et al., '48), bearing close relations to this study, has been so far unable to be repeated (ANDO and ARAKI, '56; HESS, '56; WEDDELL, '56) might give rise to a kind of encouragement to the view point.

Finally, the problem of metachromasia observed during this study and reported by WEDDELL School respectively seems, as fascinating it is, to need a complete reconsideration whenever pure methylene blue becomes available (see for references :

HELLER et al, '47; HOLMES, '28).

SUMMARY

A reevaluation of the intraarterial methylene blue, method of EHRLICH for studies of CNS was made.

1. A somewhat modified method of EHRLICH was described.
2. Major details of the results obtained by RAMÓN y CAJAL have been repeated.
3. Possibilities of the method both for a substitute of the GOLGI and as a general stain for CNS were considered.
4. Study for the central degeneration was attempted with the aim at the primary optic center. Difficulty experienced thereby was described. Necessity of further critical exploration along the line was suggested.

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Explanations for figures.

(Animals are rats unless indicated otherwise.)

Plate 1.**Fig. 1:** Pyramidal cells of various sizes, their dendrites and descending axons. Rabbit of 3 weeks old. Motor cortex. (350 ×).**Fig. 2:** Apical dendritic shafts and their collaterals. Most of them are reaching to the surface of cortex (surface upward). (370 ×).**Fig. 3:** Nerve cells and fibres of medium sizes near the basal ganglia. (310 ×).**Fig. 4:** A ganglion cell and its neighborhood. Note finer fibres communicating with others. (275 ×).**Fig. 5:** Ventral horn cells seen at cervical spinal cord. Note the surface of the upper cell with fine, end-feet like attachment. (490 ×).**Fig. 6:** A general appearance of stained neural elements under lower magnification. (250 ×). Pons, horizontal section.**Plate 2.****Fig. 7:** Striate area. Cells with dendritic connections can be followed. (275 ×).**Fig. 8:** Finest axons seen in fimbria, showing so called boutons de passages. Note their violet coloring. (490 ×).**Fig. 9:** Neighborhood of a thalamic nucleus. (350 ×).**Fig. 10:** Somewhat tortuous nerve fibres seen

in pretectum of intact animal. (310 ×).

Fig. 11: Trigeminal nerve fibres at the level of medulla. Note complicating interconnections of the fibres. (200 ×).**Fig. 12:** Fibres in the main optic tract side opposite to the lesion, showing disintegrations. 5 days postoperative. (490 ×).**Plate 3.****Fig. 13:** Somatic sensory cortex (Woolsey and Le Messurier, '48) under lower magnification. (86 ×).**Fig. 14:** Same slide showing the axon nets. (240 ×).**Fig. 15:** Axons leaving cortex at their junction with the white matter. (250 ×).**Fig. 16:** Somewhat tortuous fibres of medium size seen in the internal capsule of control animal. (670 ×).**Fig. 17:** A deeply stained degenerating fibre seen in superior colliculus 5 days after the operation. (670 ×).**Plate 4.****Fig. 18:** Some of the degenerating fibres seen at dorsal nuclei of lateral geniculate, 3 days postoperative. (540 ×).**Fig. 19:** Same slide under higher magnification. Note the vesicle formation. (1400 × oil).**Fig. 20:** Degenerating fibres of large size seen in pretectum. (600 ×).

和 文 抄 録

メチレン青の動脈内注入 (EHRlich 法)
による中枢神経系生体染色法の批判

米国セントルイス, ワシントン大学医学部精神々経学教室, マルコルム, プリス精神病院研究室

福 田 哲 雄

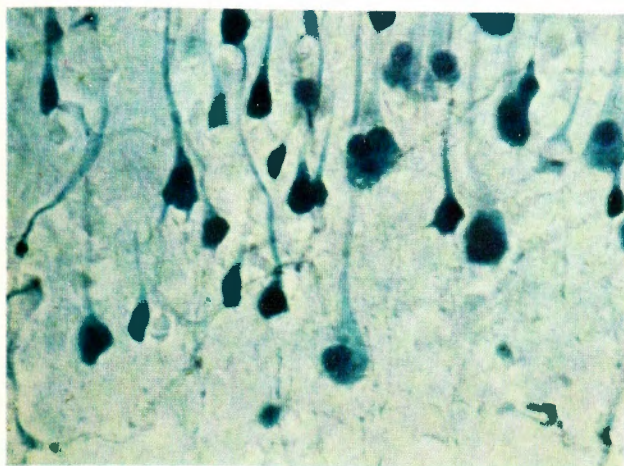
RAMÓN Y CAJAL によつて始めて中枢神経系の研究にメチレン青の動脈内注入 (EHRlich 法) が応用された (1896) が, 末梢神経系の研究に本法が広く用いられているにも拘らず, 中枢神経系に関する文献は甚だ稀である。

著者は主として体重100~200gの白鼠を用い, 2.5%メチレン青生理的食塩水液を体重100g 当り3~5cc, 頸動脈内に5~10秒間で注入する方法によつて, 本法

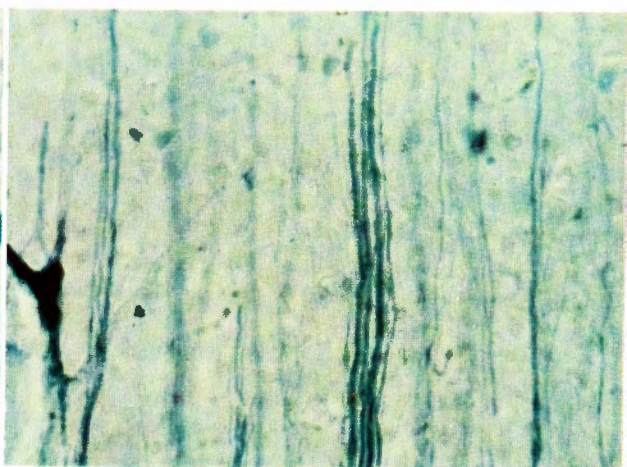
を追試し次の如き結果を得た。

1. RAMÓN Y CAJAL の得た成績の大部分が再確認された。
2. 中枢神経系の研究に対して, 本法は GOLGI 法の代用及び全景像観察用として用い得ると考えられる。
3. 中枢性変性の研究に用いるには種々困難な点があり更に検討を要する。

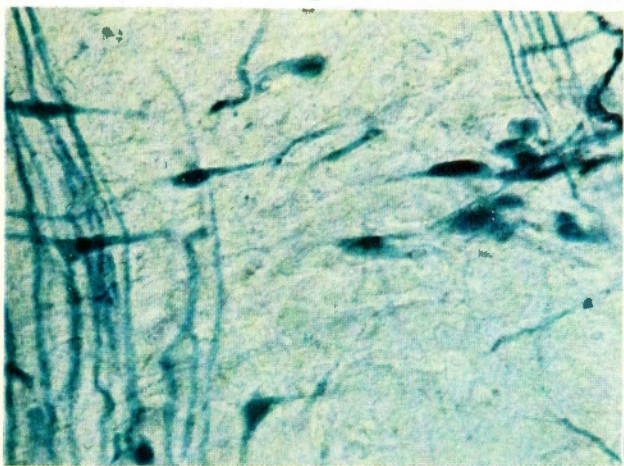
Plate 1 (Color)



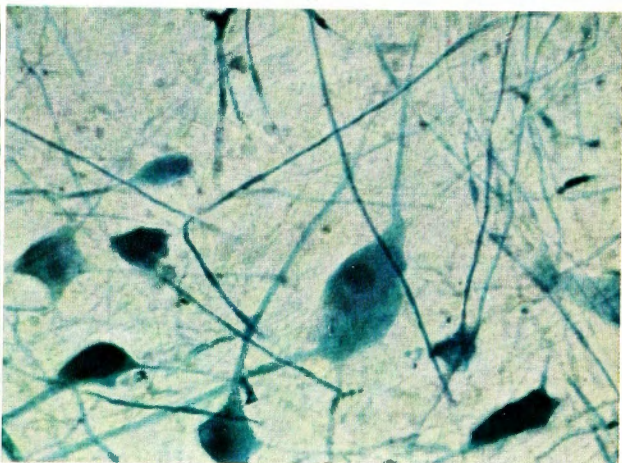
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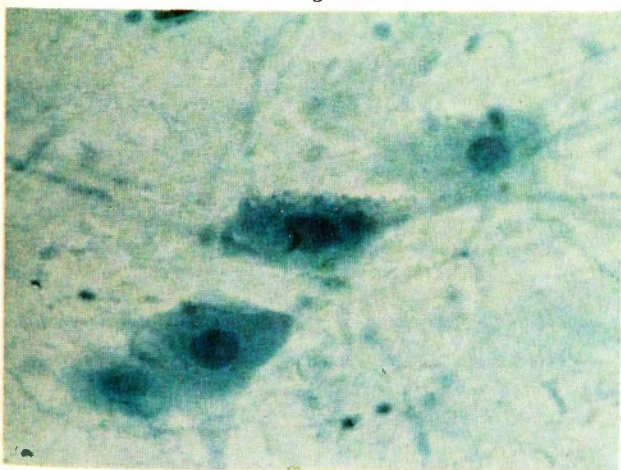
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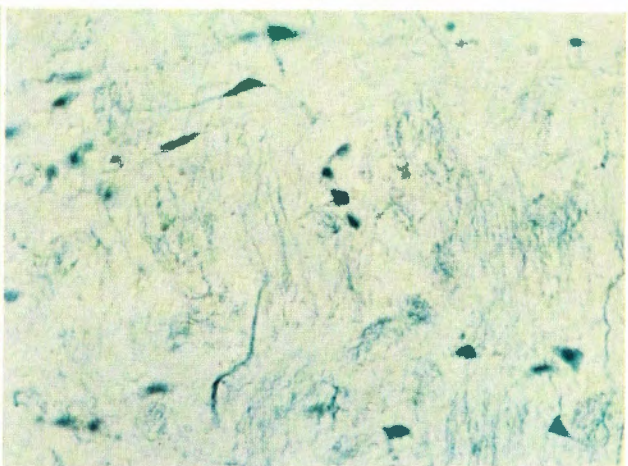
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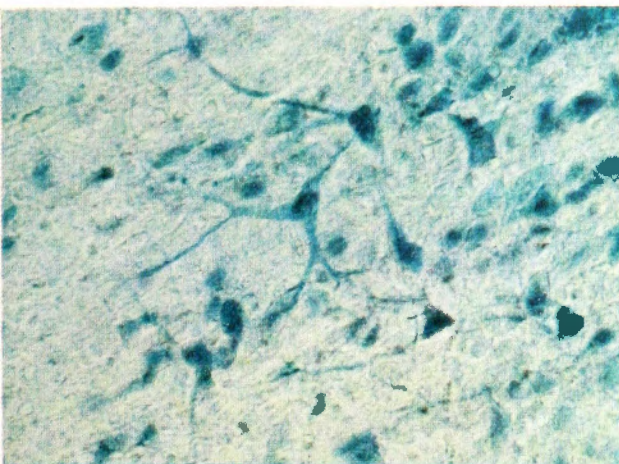


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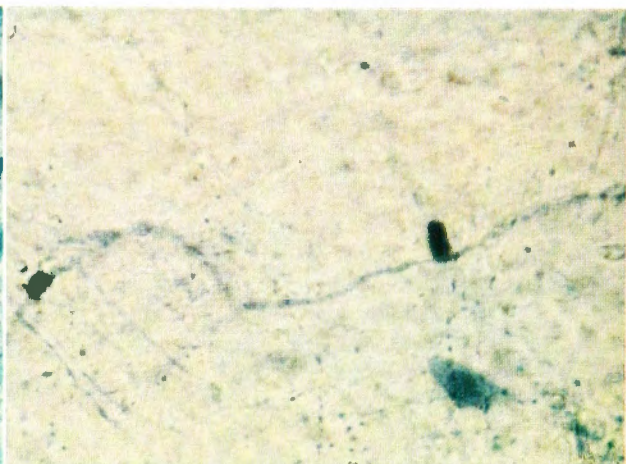


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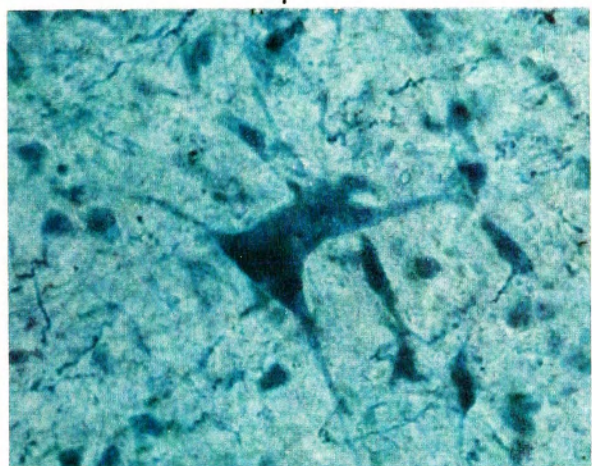
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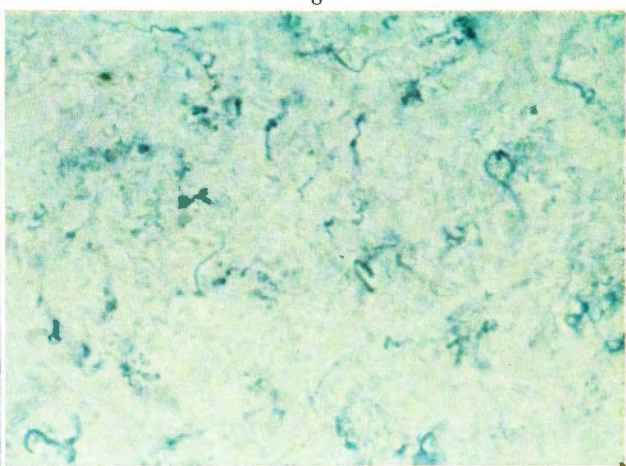
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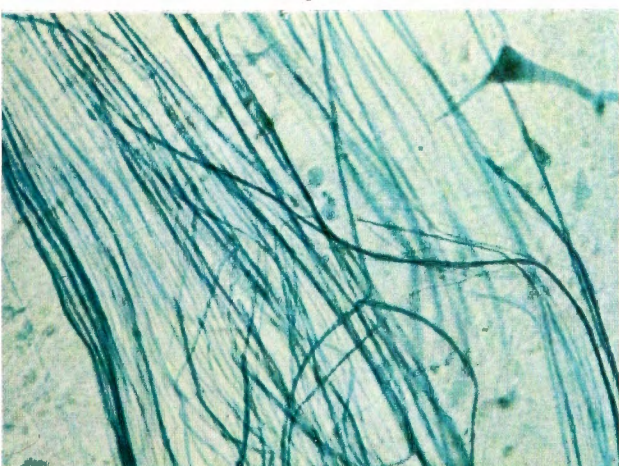
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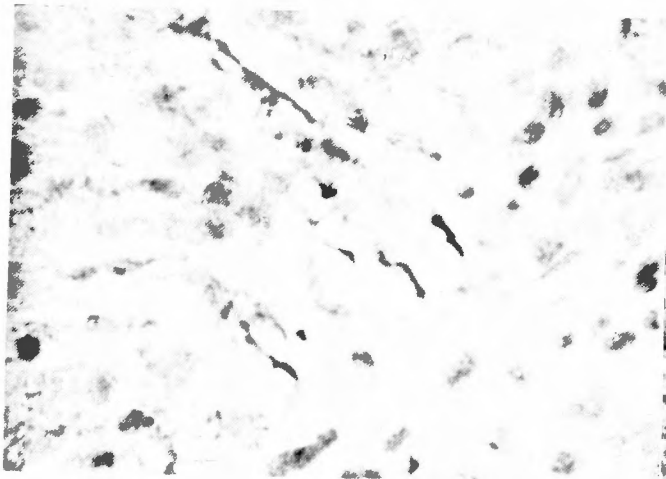
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Plate 3

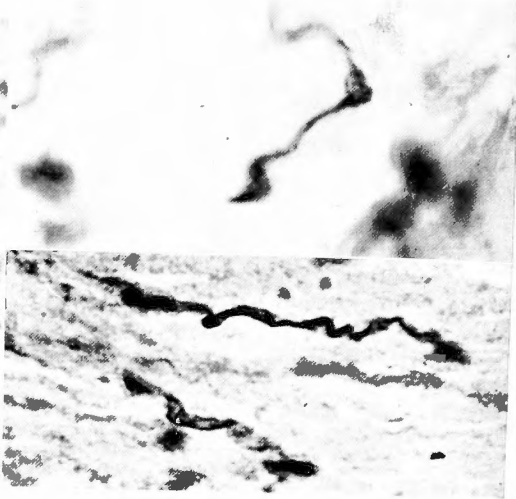


Plate 4

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